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Original Article

ORIGINAL ARTICLE

Obesity and ischemic stroke modulate the methylation levels of *KCNQ1* in white blood cells

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Abstract

Obesity and stroke are multifactorial diseases in which genetic, epigenetic and lifestyle factors are involved. The research aims were, first, the description of genes with differential epigenetic regulation obtained by an 'omics' approach in patients with ischemic stroke and, second, to determine the importance of some regions of these selected genes in biological processes depending on the body mass index. A case–control study using two populations was designed. The first population consisted of 24 volunteers according to stroke/non-stroke and normal weight/obesity conditions. The second population included 60 stroke patients and 55 controls classified by adiposity. DNA from the first population was analyzed with a methylation microarray, showing 80 cytosine-guanine dinucleotides (CpG) sites differentially methylated in stroke and 96 CpGs in obesity, whereas 59 CpGs showed interaction. After validating these data by *MassArray Epityper*, the promoter region of peptidase M20 domain containing 1 (*PM20D1*) gene was significantly hypermethylated in stroke patients. One CpG site at Caldesmon 1 (*CALD1*) gene showed an interaction between stroke and obesity. Two CpGs located in the genes Wilms' tumor 1 (*WT1*) and potassium voltage-gated channel, KQT-like subfamily, member 1 (*KCNQ1*) were significantly hypermethylated in obese patients. In the second population, *KCNQ1* was also hypermethylated in the obese subjects. Two CpGs of this gene were subsequently validated by methylation-sensitive high-resolution melting. Moreover, *KCNQ1* methylation levels were associated with plasma KCNQ1 protein concentrations. In conclusion, obesity induced changes in the *KCNQ1* methylation pattern which were also dependent on stroke. Furthermore, the epigenetic marks differentially methylated in the stroke patients were dependent on the previous obese state. These DNA methylation patterns could be used as future potential stroke biomarkers.

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Introduction

Obesity is defined as an excessive adiposity in relation to lean body mass and is usually associated with increased expression of genes related to inflammation, oxidative stress or immune response in both adipose tissue and white blood cells (1).

Ischemic stroke results from the occlusion of an afferent blood vessel and the subsequent reduction of the blood and oxygen supply to the affected brain regions (2). It accounts for roughly 80% of all clinical strokes. As for cerebrovascular diseases, stroke, either ischemic or hemorrhagic, represents the second most common cause of death worldwide and is the leading cause of long-term and permanent disability (2). There are many risk factors for stroke, including obesity, smoke habit, sedentary and raised blood pressure, total and LDL cholesterol (2–5). Besides modifiable risk factors, genetics may contribute to increase susceptibility to suffer from both obesity and stroke. However, classic genetics cannot explain the diversity of phenotypes within population disorders such as obesity or stroke (6–8). There are certain environmental factors that seem to interact with gene sequence to increase the odds of the disorders (9). In this sense, epigenetics could explain at least in part this genetic heterogeneity. Epigenetics is defined as stable and heritable patterns of gene expression and genome function that do not involve changes in DNA sequence (10). The best-known epigenetic mechanisms are DNA methylation, covalent histone modifications and miRNAs. DNA methylation is a long-term, fairly stable epigenetic modification that consists in the covalent binding of a methyl group to the 5′ carbon of a cytosine that is followed by a guanine [a cytosine-guanine dinucleotides (CpG) dinucleotide] (10,11). The main function of DNA methylation is to modulate the expression of the genetic information by modifying the accessibility of the transcriptional machinery to the DNA. There is increasing evidence that regulatory activity at the level of DNA methylation plays a role in the causation of human diseases, including cancer, diabetes, neurodegenerative disorders and obesity (12). Recent studies have demonstrated that DNA methylation levels of the tumor necrosis factor alpha (TNF-α) gene promoter are associated with metabolic features (13) and dietary response (14). The involvement of epigenetic mechanisms in gene expression regulation of stroke-related genes has been also explored in recent human studies (15). Furthermore, the role of neural cell reprogramming pathways regulated by epigenetic marks appears to hold important potential for possible innovative therapeutic approaches of brain damage (16). According to this hypothesis, some authors have suggested that, by altering transcriptional regulation, epigenetic modifiers can exert an influence on the pathways involved in the complex course of ischemic disease development (17,18). Epigenetic analysis of white blood cells, easily accessible in humans, is a promising approach for diagnosing disease (19). For example, it could help to predict the risk of age-related diseases, such as ischemic heart disease and stroke (20).

The two main goals of this study were, first, the description of genes with differential epigenetic regulation obtained by an 'omics' approach in patients with ischemic stroke and, second, to determine the importance of some regions of these differential selected genes in biological processes depending on the body mass index (BMI).

Results

To analyze and classify the screening population (SP), individuals were matched by sex and age. The differences in anthropometric and biochemical parameters between the four groups of the SP are shown in Table 1.

The results of the HumanMethylation27 Beads array showed 96 CpG sites differentially methylated between obese and non-obese subjects, 80 CpG sites differentially methylated in patients who suffered an ischemic stroke and 59 CpG sites that presented interaction between stroke and obesity. Twenty-one CpG sites from 15 genes (Supplementary Material, Data S2) were selected for further validation by taking into account

Table 1. Differences (mean ± SE) in anthropometric characteristics and biochemical parameters in the four subgroups of the SP ($n = 24$)

Variables	Non-obesity, non-stroke ($n = 6$)	Non-obesity, stroke ($n = 6$)	Obesity, non-stroke ($n = 6$)	Obesity, stroke ($n = 6$)	ANOVA Obesity	Stroke	Interaction
Gender (female/males)	3/3	3/3	3/3	3/3			
Age (years)	69.8 ± 6.9	74.6 ± 3.0	67.5 ± 5.8	66.5 ± 6.7	n.s.	n.s.	n.s.
BMI (kg/m^2)	22.8 ± 2.0	22.5 ± 1.6	34.5 ± 3.4	33.4 ± 1.1	***	n.s.	n.s.
Waist circumference (cm)	83.3 ± 12.4	84.5 ± 4.0	111.3 ± 9.3	117.5 ± 12.4	***	n.s.	n.s.
NIHSS at admission		6.0 ± 4.6		4.8 ± 6.2		n.s.	
Glucose (mg/dl)	80.8 ± 39.7	97.2 ± 20.7	86.2 ± 21.1	143.3 ± 56.5	n.s.	*	n.s.
Systolic blood pressure (mmHg)	131.6 ± 20.2	180.2 ± 20.2	145.4 ± 34.0	200.2 ± 25.0	*	*	n.s.
Diastolic blood pressure (mmHg)	80.0 ± 5.0	81.8 ± 19.3	83.0 ± 6.7	106.7 ± 16.3	n.s.	n.s.	n.s.
Total cholesterol (mg/dl)	214.8 ± 34.3	177.2 ± 28.9	200.4 ± 36.7	205.8 ± 37.3	n.s.	n.s.	n.s.
HDL-cholesterol (mg/dl)	55.4 ± 11.6	60.7 ± 17.5	52.0 ± 10.0	37.0 ± 11.2	*	n.s.	n.s.
LDL-cholesterol (mg/dl)	135.4 ± 28.2a	88.0 ± 27.3b	123.2 ± 25.8a	151.9 ± 40.1a	*	n.s.	*
Triglycerides (mg/dl)	119. ± 37.5a	86.2 ± 22.7b	125.8 ± 16.8a	164.0 ± 45.0a	n.s.	n.s.	*
Leukocytes (mil/mm^3)	5.03 ± 0.85	8.36 ± 2.07	6.55 ± 1.72	7.47 ± 2.78	n.s.	*	n.s.
Neutrophils (%)	54.63 ± 6.83	64.43 ± 10.71	55.78 ± 7.79	59.36 ± 11.13	n.s.	n.s.	n.s.
Lymphocytes (%)	31.76 ± 7.75	24.80 ± 8.49	33.45 ± 7.09	24.66 ± 13.93	n.s.	n.s.	n.s.
Monocytes (%)	9.65 ± 2.91	7.87 ± 1.89	7.98 ± 1.78	8.80 ± 2.25	n.s.	n.s.	n.s.
Eosinophils (%)	3.28 ± 2.38	2.21 ± 1.57	2.20 ± 1.57	1.38 ± 0.94	n.s.	n.s.	n.s.
Basophils (%)	0.55 ± 0.53	0.45 ± 0.43	0.42 ± 0.39	0.26 ± 0.13	n.s.	n.s.	n.s.

Two-way ANOVA test was used to analyze the interaction between obesity and stroke. LSD post-hoc was applied when possible. Values are means ± SD. n.s. non-significant. *$P < 0.05$ and ***$P < 0.001$.

CpGs with absolute methylation differences ≥5% and $P \leq 0.05$, in addition to bibliographical evidence about the relationship of these genes with obesity or stroke. Although all the selected CpG showed differences with $P < 0.05$, no array probe reached statistical significance when applying the false discovery rate correction.

The analysis of the DNA methylation patterns for the 15 candidate genes by MassArray® Epityper™ evidenced that some of these regions presented statistical differences between groups. The methylation levels of the genes Wilms' tumor 1 (*WT1*), peptidase M20 domain containing 1 (*PM20D1*), Caldesmon 1 (*CALD1*) and potassium voltage-gated channel, KQT-like subfamily, member 1 (*KCNQ1*) quantified by this technique showed a positive correlation ($P < 0.05$) with respect to the methylation levels measured by microarray studies (Supplementary Material, Data S3–S6). These results were considered as a validation between the two techniques.

In addition to a correlation with the results of the array, CpG sites 10_11_12, 19_20, 25 of the studied region of *WT1* presented a hypermethylation in the obese patients compared with the non-obese. The site 19_20 also showed higher methylation levels in the patients who had suffered a stroke, with no interaction with obesity.

In the analyzed region of PM20D1, the general trend was a hypermethylation in the patients that had suffered a stroke when compared with the non-stroke group, without interaction with BMI. Differences in this region were significant at the CpG sites 1_2_10, 11, 12, 13, 14, 16_17, 18, 22, as well as the total methylation values. CpG sites 1 and 8 of the studied region of *CALD1* showed an interaction between stroke and obesity. Although a correlation between the methylation results of the array and the MassArray® Epityper™ approach was not observed in the studied region of *KCNQ1*, the CpG sites 2 and 9 showed higher methylation levels in the obese patients when compared with the non-obese, without interaction with stroke.

For the validation of the epigenetic results obtained in the SP, the same methodological approach and primers were used in the validation population (VP; $n = 115$). The biochemical and phenotypic characteristics of VP are shown in Table 2. In this population, only CpG2 and CpG4 sites from *KCNQ1* were differentially methylated between groups (Supplementary Material, Data S7). Due to this fact, a second technical validation was performed by using a third method, methylation-sensitive high-resolution melting (MS-HRM), in 80 samples from the VP. The primers designed for this purpose flanked two regions: one equivalent to the CpG 1_2_3 measured by MassArray® Epityper™ and another equivalent to the CpG 8_9. The results (Fig. 1) showed higher methylation levels in both regions that were associated to the BMI of the patients. DNA methylation levels of CpG 8_9 also showed an interaction between stroke and obesity, with a hypomethylation in the normal weight patients who suffered an ischemic stroke. A significant and positive correlation was found between the methylation levels of the CpGs 1_2_3 and 8_9, and the BMI ($P < 0.001$; $r = 0.493$, and $P < 0.001$; $r = 0.486$, respectively) and also between the methylation levels of CPG 1_2_3 and the National Institute of Health Stroke Scale (NIHSS) ($P = 0.016$, $r = 0.371$). Finally, a lineal regression analysis was carried out in order to explain the influence of the methylation changes in both CpGs on obesity status and stroke situation and severity. Thus, both CpG sites explained a 27 and 26.5% ($p_{model} < 0.001$) of the variability observed in the BMI, 31 and 33% for the case of stroke and 43–57% in the case of NIHSS.

Table 2. Differences (mean ± SE) in anthropometric characteristics and biochemical parameters in the six groups of the VP ($n = 115$)

Variables	Normal weight, non-stroke ($n = 15$)	Normal weight, stroke ($n = 9$)	Overweight, non-stroke ($n = 15$)	Overweight, stroke ($n = 31$)	Obesity, non-stroke ($n = 25$)	Obesity, stroke ($n = 20$)	ANOVA BMI	Stroke	Interaction
Gender (females/males)	14/1	5/4	6/9	16/15	13/12	11/9			
Age (years)	60.4 ± 7.8	72.6 ± 6.4	62.8 ± 8.6	69.6 ± 7.3	62.8 ± 8.2	69.0 ± 8.9	n.s.	***	n.s.
BMI (kg/m^2)	23.0 ± 1.4	23.6 ± 1.4	27.6 ± 1.4	27.5 ± 1.6	32.7 ± 4.8	29.2 ± 4.3	***	n.s	n.s.
Waist circumference (cm)	82.7 ± 6.5	95.3 ± 8.6	97.9 ± 9.8	105.7 ± 15.1	97.8 ± 13.1	107.2 ± 15.5	***	***	n.s.
NIHSS at admission		3.1 ± 4.7		4.4 ± 4.4		4.2 ± 2.8	n.s.	n.p.	n.s.
Glucose (mg/dl)	89.7 ± 6.2	106.7 ± 35.0	98.6 ± 19.5	124.0 ± 56.4	107.2 ± 19.4	121.1 ± 2.4	n.s.	*	n.s.
Systolic blood pressure (mmHg)	124.0 ± 10.6	165.3 ± 24.2	134.4 ± 11.5	162.9 ± 26.1	133.3 ± 15.8	173.7 ± 28.6	n.s.	***	n.s.
Diastolic blood pressure (mmHg)	75.9 ± 6.6	84.7 ± 8.4	80.5 ± 6.8	85.3 ± 13.1	78.1 ± 6.2	91.3 ± 13.8	n.s.	***	n.s.
Total cholesterol (mg/dl)	229.5 ± 29.0	185.7 ± 57.8	210.1 ± 28.7	186.4 ± 37.8	213.5 ± 30.3	207.0 ± 31.8	n.s.	*	n.s.
HDL-cholesterol (mg/dl)	69.0 ± 13.8	53.2 ± 14.5	60.9 ± 13.3	44.6 ± 12.0	54.5 ± 13.3	47.8 ± 13.3	n.s.	***	n.s.
LDL-cholesterol (mg/dl)	148.7 ± 33.3	133.6 ± 27.9	131.9 ± 29.9	119.3 ± 35.1	128.5 ± 25.2	132.0 ± 31.2	n.s.	n.s.	n.s.
Triglycerides (mg/dl)	81.7 ± 30.8	79.2 ± 32.3	82.9 ± 26.7	123.6 ± 27.2	146.1 ± 83.2	166.0 ± 100.2	***	n.s.	n.s.
Leucocytes (mil/mm^3)	4.9 ± 1.1	7.5 ± 1.8	5.6 ± 1.6	7.2 ± 1.8	6.8 ± 2.1	7.8 ± 2.4	*	***	n.s.
Neutrophils (%)	51.2 ± 10.4	57.3 ± 1.3	54.1 ± 10.1	59.2 ± 10.4	57.4 ± 6.7	63.0 ± 9.1	*	*	n.s.
Lymphocytes (%)	37.4 ± 9.4	28.9 ± 9.8	34.6 ± 9.4	28.8 ± 8.6	30.1 ± 6.3	26.2 ± 7.2	*	*	n.s.
Monocytes (%)	8.1 ± 2.5	7.8 ± 2.0	7.4 ± 1.4	8.7 ± 2.3	8.1 ± 1.8	7.8 ± 2.1	n.s.	n.s.	n.s.
Eosinophils (%)	2.3 ± 1.1	2.6 ± 2.2	2.7 ± 1.5	2.6 ± 2.1	3.3 ± 1.4	1.7 ± 1.	n.s.	n.s.	n.s.
Basophils (%)	0.5 ± 0.3	0.4 ± 0.2	0.6 ± 0.4	0.4 ± 0.3	0.6 ± 0.3	0.3 ± 0.2	n.s.	n.s.	n.s.

Two-way ANOVA test was used to analyze the interaction between obesity and stroke. Values are means ± SD. n.s. non-significant. *$P < 0.05$ and ***$P < 0.001$.

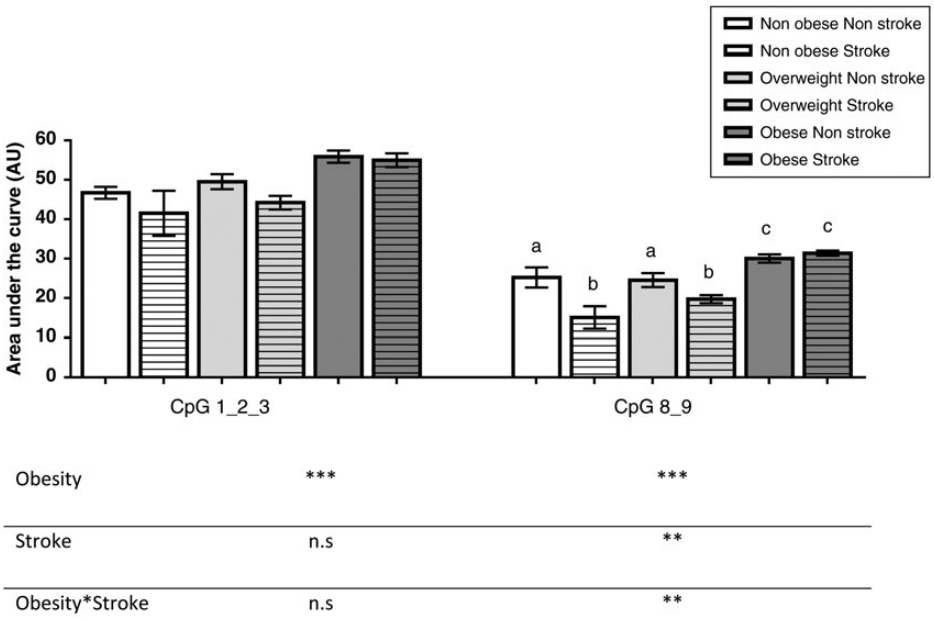

Figure 1. Methylation levels (area under the curve) of CpG 1_2_3 and 8_9 of *KCNQ1* region as measured by MS-HRM in the VP ($n = 80$); n.s: non-significant. $^{**}P < 0.01$, $^{***}P < 0.001$. Two-way ANOVA test was used to analyze the interaction between obesity and stroke. Different letters indicate significant differences between groups from at least $P < 0.05$ using a LSD post-hoc test if applicable.

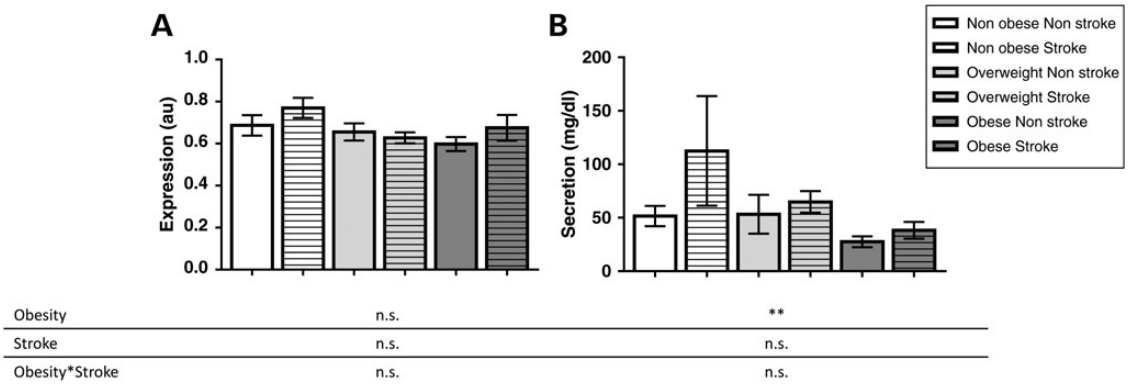

Figure 2. (**A**) Gene expression of *KCNQ1* in total white blood cells from the SP ($n = 101$). (**B**) Plasma levels of KCNQ1 in the VP ($n = 80$); n.s: non-significant. $^{**}P < 0.01$. Two-way ANOVA test was used to analyze the interaction between obesity and stroke.

These changes were also noted in the plasma levels of KCNQ1. Significant differences were observed in the obese patients when compared with the normal weight and overweight subjects (–37%, $P = 0.006$). The methylation changes in the SP were also associated with the variations in *KCNQ1* gene expression, with an interaction between obesity and stroke (Fig. 2). Finally, no differences were found in *PM20D1*, *WT1* and *CALD1* methylation levels in the VP.

Discussion

The hypothesis of the present work was that the methylation pattern of some epigenetically regulated genes could change after stroke and that these changes are mediated by the previous body composition. The phenotypic data from both populations (SP and VP) were similar. Besides the differences in BMI derived from the selection of the different groups, significant differences were observed in the waist circumference between stroke and non-stroke patients. These data are consistent with those of other authors, who determined that the waist circumference is one of the major risk factors for stroke (21,22). In both populations, plasma glucose levels were higher in the stroke patients regardless of BMI. These data are consistent with those described by Rundek *et al.* in a cohort of more than 1500 non-diabetic individuals (23), in which they found that glucose and the homeostatic model assessment index were markers of increased risk of incident stroke in non-diabetic individuals. Moreover, post-stroke hyperglycemia is a common phenomenon, with up to 50% of patients in two-thirds of ischemic stroke subtypes having blood glucose levels of above 108 mg/dl (24). There is evidence to suggest that hyperglycemia is detrimental in cerebral ischemia (25). These biochemical changes may explain changes in

methylation patterns in patients who suffered an ischemic stroke, as in obese or overweight patients. Interestingly, it has been hypothesized that hyperglycemia may be mediated by the promoter hypomethylation of the insulin gene, inducing a lower secretion of the hormone (26). However, we have not found correlation between plasma glucose levels and the DNA methylation levels of the different CpG sites analyzed.

Another phenotypic alteration that could be influenced by obesity and stroke is the blood cell distribution. In both populations, leukocyte number was higher in stroke patients. These results can be explained by the rupture in the blood–brain barrier that allows immune system cells to gain access to the brain after an ischemic stroke accident (27). A large number of stroke patients in clinical studies showed a significant elevation in total leukocyte and polymorphonuclear leukocyte (PMNL) count in peripheral blood after ischemia (28–30). In our study, this increase in PMNL was confirmed in the stroke patients, with higher percentage of neutrophils, lymphocytes and monocytes in the VP. Also only in the VP, differences in the number of leukocytes, neutrophils and lymphocytes were found between obese and non-obese patients. A similar process has been described in obesity, with more total white cells and leukocytes as a consequence of increased low-grade inflammation resulting from an increase in adipose tissue mass (31). In other studies, the number of leukocytes has been correlated with leptin levels, insulin resistance, lipid profile and adiposity (32,33).

Epigenetic marks, especially DNA methylation, may play an important role in cellular differentiation and gene regulation (34) and have been linked to different pathological conditions like cancer (12). These changes in epigenetic marks could also be related to the risk of developing obesity and ischemic stroke. For example, previous studies of our group have described that obesity, weight loss and dietary response are mediated by epigenetic changes (14,35,36). On the other hand, high fat and sucrose diets induce changes in the DNA methylation pattern of genes involved in appetite regulation in the hypothalamus (37). However, the effect of the epigenetic marks on stroke outcome has not been extensibility studied. In this sense, Kim *et al.* analyzed the possible association of a hypermethylation in the *BDNF* promoter region in leukocytes with neurological worsening at 1 year, and with the worsening of physical disability and cognitive function (38).

In order to research for more widely epigenetic mechanisms involved in stroke and its relationship to obesity and overweight, a methylation array was performed in the PS. A total of 96 CpGs were modified in obese patients, 80 CpGs in stroke and 59 CpGs presented an interaction. From them, 21 CpG from 15 regions of genes were selected for validation by the *Epityper* approach, and the methylation patterns of four regions from four genes (*WT1*, *CALD1*, *PM20D1* and *KCNQ1*) showed similar results with the two techniques. A similar validation approach has been previously used by our group (36,39) and by other authors (40).

WT1 is a gene involved in the development of the Wilms' tumor aniridia, genitourinary and mental retardation syndrome, which in many cases is accompanied by obesity (41). *WT1* methylation has been extensively tackled in cancer research, but our group found changes in its methylation pattern after a nutritional treatment, suggesting a possible implication of *WT1* in obesity (36). As in that study, the current has found a hypermethylation in the obese patients when compared with the non-obese individuals. However, the patients that suffered a stroke also showed hypermethylation in the CpG site 19_20, without interaction with obesity.

CALD1 encodes a calmodulin- and actin-binding protein that plays an essential role in the regulation of smooth muscle and non-muscle contraction. This gene is involved in the contraction and relaxation of arterial muscle (42). This study is the first that analyzes the methylation levels of *CALD1* and its possible implication in obesity and stroke. In this gene, two CpG sites (CpG 1 and CpG8) showed an interaction between stroke and obesity.

The region analyzed at the metalloproteinase gene *PM20D1* was selected due to the high differences between stroke and non-stroke subjects found in the methylation array. These differences were later observed in several CpGs in the SP. Although, up to now, the function of *PM20D1* is unknown, several studies have found differences in the *PM20D1* methylation levels in whole blood (43,44), suggesting that the methylation of this gene could be used as a biomarker in different situations.

KCNQ1 is an imprinted gene that encodes a voltage-gated potassium channel required for the repolarization phase of the cardiac action potential. Several SNPs along this gene have been linked to diabetes development (45). Interestingly, *KCNQ1* mutations increase the risk of cardiac events with physical or emotional stress (46). Despite the evidence found, our results showed differences in the methylation levels of two CpG sites in the obese patients when compared with non-obese in the SP.

Only for *KCNQ1* methylation data in the VP correlated with the methylation levels in the SP. This is the most important result of this study: stroke and obesity modulated the DNA methylation pattern of *KCNQ1* in both populations. A possible explanation for the lack of validation of *WT1*, *PM20D1* and *CALD1* regions may be the specificity of DNA methylation in the different cell types. Studies on DNA methylation using whole blood DNA frequently do not control inter-individual variation in the cellular population from which the DNA is derived (lymphocytes, neutrophils, eosinophils, basophiles and monocytes). This approach has been criticized due to hypothesized potential for confounding effect when cellular heterogeneity is present in conjunction with cell type specific DNA methylation (47,48). Thus, Adalsteinsson *et al.* showed a correlation between the methylation of *PM20D1* and changes in the percentage of methylation in different types of white blood cells. This author suggested that the use of whole blood for epigenetic studies is a confusing factor that increases the inter-individual variability. These arguments could explain at least in part the differences of up to 20% that we have observed in several *PM20D1* CpG sites in the SP, whereas no differences were found in the VP. As discussed above, differences were found in the different cell types present in the total white cells. In any case, many articles have previously demonstrated the utility of white blood cells, and particularly the leukocytes, as a source of DNA methylation biomarkers, as well as the impact of the epigenetic variations on common complex diseases (40,49).

Concerning the lack of reproducibility between different populations when using the same technique, in this case MassArray® EpiTyper™, it could be explained by differences in sample extractions. In this sense, it is known that different salt concentrations in the samples could produce salt adducts that can generate spectral peaks at their corresponding molecular weights that may be observed after mass spectrometry and potentially interfere with the analysis (50). Another issue that might explain the lack of reproducibility could be the pharmaceutical heterogeneity that existed among populations. SP population had pharmacological uniform registration, while the VP population received a huge heterogeneity of drugs. This variable has been taken into account in some analyses as a confounding factor.

Surprisingly, DNA methylation levels were associated with protein concentration of KCNQ1, but not with *KCNQ1* mRNA levels in white cells. Some hypotheses may explain this fact, including the different life span of both mRNA and protein, CpGs sites that are not directly related to a classic region of epigenetic regulation (i.e. CpG islands), CpGs sites specific to transcription factors that can directly modify the activity of other transcription factors or the regulation of protein expression at different levels (transcriptional, post-transcriptional, post-translational or post-transductional signal). So, epigenetic marks and protein secretion could be more stable and better indicators of the patient's clinical status than the mRNA levels, which are more variable and change more rapidly in response to environmental and hormonal stimuli.

KCNQ1 region was analyzed with the Genomatix platform (MatInspector application) in order to find a potential consensus sequence for transcription factors, which could be a possible mechanism affected by DNA methylation. We have observed that the methylation of specific CpG sites of the *KCNQ1* promoter could be potentially regulating HES1 response elements, E-box and HIF-1 functions, all of them previously associated to stroke (51–54), which should be investigated in more detail in stroke and neural cells, as well as in obesity, adipogenesis and lipid and glucose metabolism.

Finally, the ultimate goal of this study was the search of stroke prognostic biomarkers by using epigenetic marks in a non-invasive sample (buffy coat). This prognosis will be faster than the NIHSS and can complement it for a better prognostic assessment of patients with stroke. For a better treatment of stroke, prognosis must be personalized (by age, sex, and, of course, by the metabolic state, including obesity, that may impact the sensitivity and reproducibility of the biomarkers found). Therefore, an important derivative of this paper is that we have not only found an epigenetic biomarker associated with stroke, but that it can be implemented in clinical practice depending on the patient's BMI, which is a first step toward personalized prognosis of stroke.

In conclusion, the methylation levels of a region of the *KCNQ1* promoter could be used as a biomarker of diagnosis of ischemic stroke, although always depending on the obesity condition of the patients.

Materials and Methods

Study population

The study consisted in a case–control design with two populations (Supplementary Material, Data S1). In both populations, cases were selected from the Neurology Service of Hospital Donostia, with a first episode of ischemic stroke, evaluated in the first 24 h for a neurologist of the onset of symptoms. NIHSS (55) was carried out to stroke patients at the time of hospital admission, in order to determine and measure neurologic deficits. The control groups were constituted by non-vascular neurological disorder patients that were matched by sex and age with the cases.

The SP included six obese and six non-obese subjects in each group, matched also by BMI and waist circumference with cases. Non-obese groups were defined by a BMI <25 kg/m^2 and a waist circumference <88 cM in women and <92 cM in men. Obese groups included subjects with a BMI >30 kg/m^2 and a waist circumference >88 cM in women and >92 cM in men.

The VP included 115 voluntaries, who were categorized by BMI according to WHO classification in normal weight (BMI ≤25 kg/m^2), overweight (BMI 25.1–29.9 kg/m^2) and obesity (BMI ≥30 kg/m^2). Exclusion criteria were age lower than 50 years old and higher than 80 years old in both groups. In stroke groups, those patients having suffered a hemorrhagic stroke or another cerebrovascular disease different to ischemic stroke were excluded. The control group did not include patients having suffered a vascular or neurological disorder.

A written informed acceptance was obtained from all participants or by a direct familiar in the case of severe disability, in agreement with the Helsinki Declaration. The study protocol and informed consent document were approved by the corresponding Medical Ethical Committee (act 2/10 of the Comité Ético de Investigación Clínica del Área Sanitaria de Gipuzkoa).

Anthropometric measurements, acid nucleic isolation and biochemical determinations

For all voluntaries, anthropometric parameters were measured by standard methods. BMI was calculated as weight/height^2. Peripheral blood samples were extracted in the first visit in control volunteers and in the emergency service in the stroke patients. Buffy coat and plasma were extracted from peripheral blood and subsequently stored at −80°C. DNA was extracted using the Qiagen Flexigen kit (Qiagen, Hilden, Germany). DNA concentrations were determined with a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). PerfectPure RNA Blood Kit (5 Prime, Hamburg, Germany) was used for RNA isolation from white blood cells. Glucose, total, HDL and LDL cholesterol, triglycerides and hemogram (leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophiles) were analyzed with a Pentra C-200 autoanalyser (HORIBA ABX, Madrid, Spain) following standardized procedures. Blood pressure was assessed according to the World Health Organization criteria (56). Plasma concentration of KCNQ1 was quantified by a specific ELISA as described by the manufacturer (Cusabio, Wuhan, Hubei, China).

DNA methylation

To identify candidate genes with different methylation profiles, 500 ng of DNA isolated from buffy coat of the SP ($n = 24$) was analyzed by the Illumina methylation 27 BeadChip array (Illumina, San Diego, CA, USA). Bisulfite modification of genomic DNA (500 ng for Illumina array and MALDI-TOF approaches) was carried out using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Bisulfite-treated genomic DNA was whole-genome amplified, hybridized to HumanMethylation27 BeadChips (Illumina, USA) and scanned using the Illumina iScanSQ platform. The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v. 1.9.0, Illumina, USA) and the background was calculated from negative control probes with this Software. The data were quality-controlled and background-corrected. β-values were computed using the formula β-value = M/[U + M], where M and U are the raw 'methylated' and 'unmethylated' signals, respectively.

β-values were imported and normalized using a categorical Subset Quantile Normalization (SQN) method with the Array Studio software (Omicsoft Corporation, Research Triangle Park, NC, USA). Probes associated with X and Y chromosomes were removed from the analysis. To identify CpG sites differentially methylated in stroke and/or obesity samples compared with healthy individuals, the limma package (57) for the Array Studio statistical software was used to compute a moderated *t*-test. Raw *P* values were adjusted using the Benjamini–Hochberg procedure

(58) and $P \leq 0.05$ was used as statistical significant threshold. Fifteen CpG candidate sites ($P < 0.050$; Δ methylation > 5%) identified from the microarray were subsequently validated in SP by a MALDI-TOF mass spectrometry-based method, *Sequenom MassArray® EpiTyper™* approach (Sequenom, San Diego, CA, USA) after designing specific primers. Those CpG sites showing positive correlation in the microarray and the EpiTyper approach were selected for validation within the VP by using another technique, MS-HRM (59).

Gene expression

DNase treatment was performed with a DNA-free™ kit (Applied Biosystems, Austin, TX, USA), while cDNA was synthesized using M-MLV reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed following manufacturer's recommendations using ABI PRISM 7900 HT Fast Real-Time PCR System and pre-designed Taqman probes for human genes (Applied Biosystems). cDNA levels were normalized with two housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Hs03929097_g1) and *18s rRNA* (Hs03003631_g1) by using Genorm (60). Fold changes between groups were calculated by using the $2^{-\Delta\Delta Ct}$ method (61).

Promoter analysis

To predict putative *cis*-acting elements in the KCNQ1 promoter, MatInspector version 9.0 was used, with 0.80 of core similarity and optimized matrix similarity as the setting parameters (http://www.genomatix.com) (62).

Statistical analysis

In order to analyze BeadArray data and to assign site-specific DNA methylation values to each CpG site, Illumina's Genome Studio software (www.openbioinformatics.org/penncnv/penncnv_beadstudio_tutorial) was used. The proportion of methylation (%) for each subject at each CpG site was computed by subtracting the background signal intensity of negative controls from both the methylated and unmethylated signals and then dividing the ratio of the methylated signal intensity by the sum of both methylated and unmethylated signals. Thus, the percentage value is a continuous variable ranging between 0 and 1. Statistical analysis was performed using ArrayStudio (OmicSoft Corp., Research Triangle Park, NC, USA). Data are shown as mean ± SD. Differences in the diagnosis groups and potential interaction between body composition and stroke were analyzed by a two-way ANOVA (obesity, stroke, obesity × stroke, or BMI, stroke and BMI × stroke), followed by a least significant difference (LSD) test for multiple comparisons. A lineal regression analysis was performed to explain the variation of related variables. The associations were calculated with Pearson's correlation coefficient. All analyses were performed with SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). *P*–values <0.05 were considered statistically significant.

Data repository

Methylation microarray data are available in the ArrayExpress database (63) (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2344.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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